

African Journal of Biotechnology Vol. 10(55), pp. 11696-11702, 21 September, 2011

Available online at <http://www.academicjournals.org/AJB>

DOI: 10.5897/AJB11.577

ISSN 1684-5315 © 2011 Academic Journals

*Full Length Research Paper*

# DNA damage protection and 5-lipoxygenase inhibiting activity of areca (*Areca catechu* L.) inflorescence extracts

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Accepted 27 July, 2011

**DNA damage caused by free radical is associated with mutation-based health impairment. The protective effect on DNA damage mediated by hydroxyl radical and peroxynitrite radical, and the inhibiting activity on 5-lipoxygenase of areca inflorescence extracts were studied *in vitro*. The results show that the boiling water extract significantly restrain the oxidative damage of pBR322 plasmid DNA induced by free radical, and synergize with trolox. Also, the inhibiting activity of boiling water extract on 5-lipoxygenase was higher than that of trolox. The antioxidant evaluation shows that the boiling water extract has the strongest inhibiting activity on  $\beta$ -carotene autooxidation and the scavenging activity on ABTS radical cation. The total phenolics concentration for boiling water extract was 5.81 mg GAE/g.**

**Key words:** Areca inflorescence extracts, trolox, DNA damage, antioxidant.

## INTRODUCTION

Epidemiological, biological and clinical studies have provided a growing number of evidences in the past decades to demonstrate that oxidative DNA damage mediated by reactive oxygen and nitrogen species is intimately associated with mutagenesis, aging and carcinogenesis (Bjelland and Seeberg, 2003; Moriwaki et al., 2008; Barja, 2004; Olinski et al., 2002). The hydroxyl radical generated by Fenton reaction when iron salts are incubated with hydrogen peroxide will cause DNA damage and 8-hydroxydeoxyguanosine (8-OHdG) formation (Tenopoulou et al., 2005; Gleit et al., 2002). Furthermore, peroxynitrite ( $\text{ONOO}^-$ ), a production of nitric oxide (NO) reacting with superoxide radical ( $\text{O}_2^{\bullet-}$ ), could induce peroxidation of lipids, oxidation of sulfhydryl group in protein, and damage of single and double DNA strand (Darley-Usmar and Halliwell, 1996; Spencer et al., 1996). Recent data have further provided evidence *in vivo* that

$\text{ONOO}^-$  were formed in human atherosclerosis, mitochondria dysfunction, neurodegeneration and chronic inflammation (Kaur and Halliwell, 1994; Yamamoto et al., 2002; Ischiropoulos and Beckman, 2003; Kooy et al., 1995). Thus, it is necessary to develop some drugs which can help to protect DNA damage caused by free radicals.

Areca nut (*Areca catechu* L., *Palmyraceae*) is an important southern medicine in China. Areca inflorescence, a potential antioxidant which is rich in polyphenolic compounds, is consumed as a material of functional food in southern China. The areca inflorescence extracts (AIEs) of boiling water extracts has antioxidant properties, inhibits 2-deoxyribose degradation, inhibit the activity of tyrosinase and has protective effect on human serum albumin damage induced by hydrogen peroxide (Chunmei et al., 2010; Fangfang et al., 2010; 2011). This study was designed to investigate the protective effects of two AIEs, and their synergistic effects with trolox on DNA damage caused by hydroxyl radical and peroxynitrite radical, 5-lipoxygenase inhibiting activity, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization and  $\beta$ -carotene

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autoxidation were also investigated *in vitro* to make clear the potential mechanism and to provide a theory basis for the exploitation and utilization of areca inflorescence products. The results demonstrate that areca inflorescence is a good radical scavenger and a protective agent that protects DNA from damage which indirectly restrains the formation of mutation based health impairment.

## MATERIALS AND METHODS

Trolox and ABTS were obtained from Sigma Chemical Co. (ST. Louis, MO, USA). pBR322 and EB were obtained from Takara and  $\beta$ -carotene, linoleic acid and Tween 40 were purchased from TCI (Tokyo, Japan). Other common reagents were purchased from Aladdin Chemical Co. (Shanghai, China). All other chemical were of the highest quality available.

### Preparation of AIEs

Areca inflorescences were sourced from Wenchang city, Hainan Province and respectively extracted three times with boiling water for 30 min and ambient water for 6 h with the ratio of solid to liquid of 1:10. Then, filtrates were combined and centrifuged at room temperature and 12000 *g* for 30 min.

The extracts were dried via rotary evaporation method, fixed to the same volume and then yielded two extracts: boiling water extract (BWE) and ambient water extract (AWE). The stock solutions were kept at 4°C in the dark until further analysis.

### Determination of total phenolics concentration

The concentration of total phenolics was determined by the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). Measurements were carried out in triplicate and calculations were based on a calibration curve obtained with gallic acid. The total phenolic content of the extract samples was expressed as gallic acid equivalent (GAE mg gallic acid/g areca inflorescences).

### Assay for hydroxyl radical induced oxidative DNA strand breaks

DNA strand breaks by hydroxyl radical were assayed by measuring the relaxation of supercoiled plasmid to an open circular form (Zheng et al., 2006). To assay this damage, reaction mixtures (25  $\mu$ l) containing 100 mM sodium phosphate buffer (pH 7.4), pBR322 DNA (0.25  $\mu$ g), FeSO<sub>4</sub> (0.5 mM), H<sub>2</sub>O<sub>2</sub> (0.2 mM) and samples were prepared. After incubation at 37°C in water bath for 1 h, the reaction liquid were mixed with 5  $\mu$ l of gel loading buffer (0.25% bromophenol blue and 40% (w/v) sucrose), then the reaction mixtures were loaded onto a 1% agarose gel stained with GoldView, and immediately electrophoresed in a horizontal slab gel apparatus in TAE buffer (40 mM Tris, 20 mM sodiumacetate and 2 mM ethylenediaminetetraacetic acid) at 90 V for 50 min. The gels were photographed on UV transilluminator.

### Inhibitory effects on supercoiled DNA breakage induced by peroxynitrite

#### Synthesis of peroxynitrite

A solution of 0.7 M H<sub>2</sub>O<sub>2</sub> (10 ml), 0.6 M HCl (10 ml) and 0.6 M

NaNO<sub>2</sub> (10 ml) were chilled to 4°C in an ice/water mixture, and 20 ml of 1.5 M NaOH was added with gentle mixing in order to quench this reaction (Uppu and Pryor, 1996; Ziogas and Tanou, 2010). Then, the unreacted H<sub>2</sub>O<sub>2</sub> was then removed by passing the aqueous phase through a 0.5  $\times$  5 cm column filled with 0.25 g of granular MnO<sub>2</sub> immediately. To minimize the dilution of peroxynitrite in the aqueous phase, the first few microlitres of the peroxynitrite solution eluting from the MnO<sub>2</sub> column were discarded. The peroxynitrite concentration was assayed by diluting the solution 1000-fold with 0.1 M NaOH and then measured at 302 nm ( $\epsilon$  = 1670 M<sup>-1</sup>cm<sup>-1</sup>).

### Assay for peroxynitrite-induced DNA nicking

In a total volume of 25  $\mu$ l, the 0.5 mM peroxynitrite was added in a reaction mixture containing 100 mM sodium phosphate buffer at pH 7.4, 0.1 mM diethylenetriaminepentaacetic acid (DTPA), 0.25  $\mu$ g of intact pBR322 plasmid DNA (Barr and Gedamu, 2003) and 10  $\mu$ l extracts. After incubation at 37°C in water bath for 1 h, the DNA bands were separated on a 1% (w/v) agarose gel and analyzed as described in previous experiment.

### The ABTS radical cation decolorization assay

ABTS was dissolved in water to make a concentration of 7 mM. ABTS<sup>+</sup> was produced by reacting the ABTS stock solution with potassium persulfate which final concentration was 2.45 mM and allowing the mixture to stand in the dark at room temperature for 12 to 16 h before use (Erel, 2004). For the study of samples, the ABTS<sup>+</sup> stock solution was diluted with 100 mM sodium phosphate buffer at pH 7.4 to an absorbance of 0.70 at 734 nm. After the addition of 2.0 ml of diluted ABTS<sup>+</sup> to 10  $\mu$ l of the samples on different concentrations, the absorbance reading was taken 5 min after the initial mixing.

### Assay for $\beta$ -carotene autoxidation

Oxidation of linoleic acid was measured by the method described by Alessandra braca (2003) with some modifications. A solution of 5 mg of  $\beta$ -carotene in 10 ml of CHCl<sub>3</sub> was placed in a beaker, and then quantities of linoleic acid (250  $\mu$ l) and Tween 20 (2.5 ml) were added. After evaporation of CHCl<sub>3</sub> at 45°C in a water bath, 500 ml of distilled water saturated with oxygen for 30 min were added. To 2.5 ml of this mixture, 500  $\mu$ l of the samples were added. Samples without test compounds were used as the blanks. Samples were subjected to oxidation by placing in an oven at 50°C for 2 h. The absorbance was read at 470 nm at regular intervals. The antioxidant activity was expressed as AA and calculated with the equation:

$$AA (\%) = 100[1 - (A_0 - A_t)/(A_{00} - A_{0t})]$$

Where, A<sub>0</sub> is the absorbance at the beginning of the incubation with compounds; A<sub>t</sub> is the absorbance at time t with compounds; A<sub>00</sub> is the absorbance at the beginning of the incubation without compounds; and A<sub>0t</sub> is the absorbance at time t without compounds.

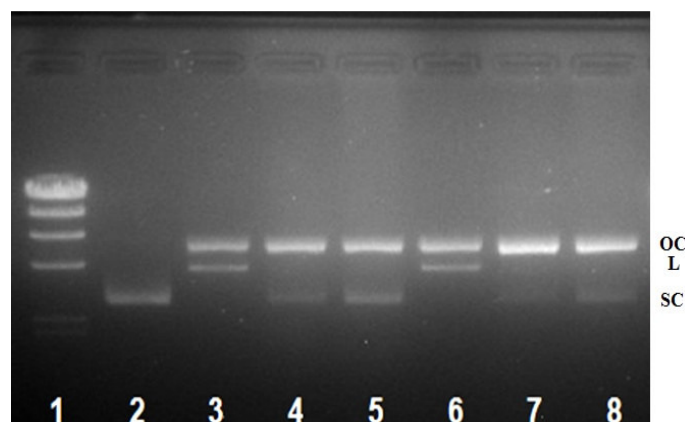
### Lipoxygenase inhibitory activity of AIEs

Lipoxygenase assay was performed as previously reported by Sajid and Soottawat (2010) with a slight modification. To study the inhibitory activity towards soybean lipoxygenase, 10  $\mu$ l of different phenolic compounds at various concentrations (10, 25, 50 and 100 mg/L) were mixed with 5  $\mu$ l of 0.1 M sodium borate buffer solution

**Table 1.** Yields of areca inflorescence extracts and their total phenolics concentration (TPC).

Extract	Weight of extracts (mg/g)	TPC (mg GAE/g)
AWE	132.20±0.76 <sup>b</sup>	5.02±0.01 <sup>b</sup>
BWE	150.13±0.94 <sup>a</sup>	5.81±0.04 <sup>a</sup>

Each value is expressed as mean ± SD. Means followed by the different letter within each line are significantly different ( $P < 0.05$ ;  $n = 3$ ).



**Figure 1.** Protect effects of AIEs and trolox on •OH induced plasmid pBR 322 DNA damage. Lane 1, Hind III λ-digest DNA size markers; lane 2, intact pBR322 DNA (no Fenton reagents); lane 3, Fenton reactants-induced DNA scission (no extracts); lane 4, •OH + AWE; lane 5, •OH + BWE; lane 6, •OH + trolox; lane 7, •OH + AWE + trolox; lane 8, •OH + BWE + trolox; SC, supercoiled DNA; OC, open circular DNA; L, linear DNA.

(pH 9.0) containing lipoxygenase (150 U). The mixtures were allowed to stand at 25°C for 10 min, followed by the addition of 2.97 ml of 0.1 M sodium borate buffer (pH 9.0). To initiate the reaction, 20 µl of 3 mM linoleic acid were added. The resultant solution was mixed well, and the linear increase of absorbance at 234 nm was measured after 5 min. One unit of lipoxygenase was defined as the increase in 0.1 unit of absorbance at 234 nm/min. The percentage inhibition was calculated as follows:

$$\text{Lipoxygenase inhibition (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where,  $A_0$  is the activity without inhibitor and  $A_1$  is the activity in the presence of inhibitor.

### Statistic analysis

Data were reported as mean ± SD for triplicate determinations. Analysis of variance and least significant difference were assessed by one-way ANOVA tests (SPSS 11.5) which were conducted to identify differences among means. Statistical significance was declared at  $P < 0.05$  and graphs were presented by using OriginPro 8.0 package.

## RESULTS AND DISCUSSION

### Total phenolics concentration of AIEs

Table 1 shows the yield of AIEs and their total phenolic

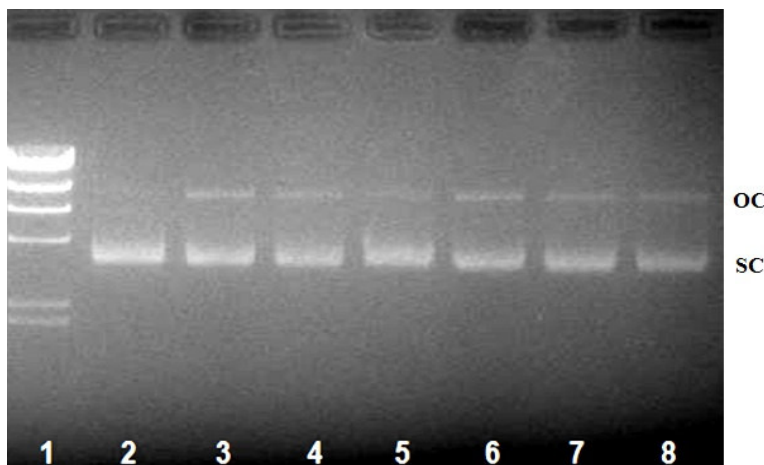
concentrations which was considered as a major decisive factor for the antioxidant activity of nuts and plants (Kris-Etherton et al., 2002). It revealed that the yields of BWE and AWE were 150.13 and 132.20 mg/g, respectively. Furthermore, the total phenolics concentration of BWE (5.81 mg GAE/g) was higher than that of AWE (5.02 mg GAE/g) indicating that the boiling water is more efficacious in extracting phenolic compounds from areca inflorescence than ambient water.

### Effects of DNA strand break induced by hydroxyl radicals

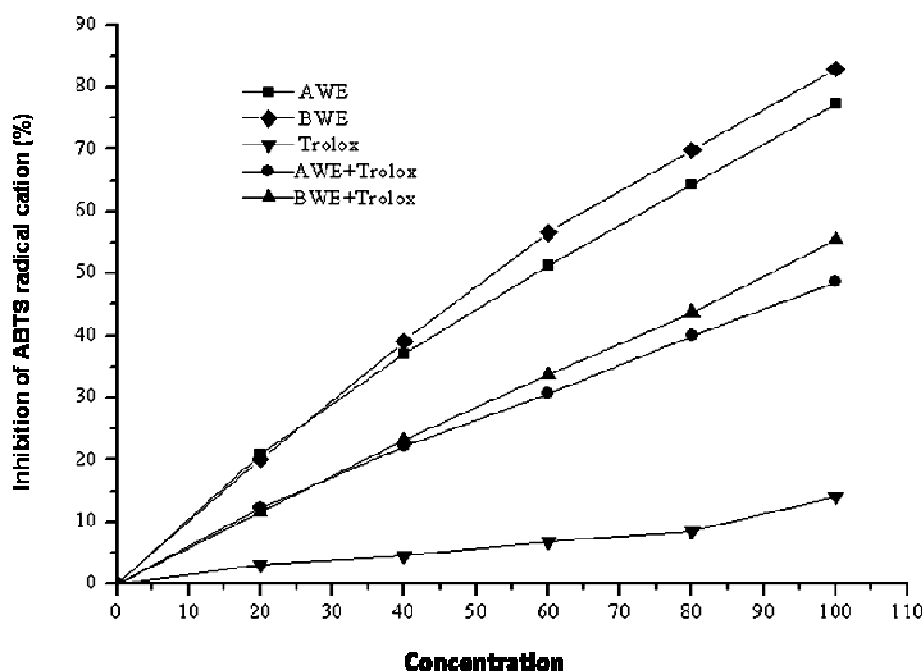
Hydrogen peroxide is commonly reduced *in vivo* by iron (II), which leads to the formation of hydroxyl radical via the Fenton reaction (Henle et al., 1999). Biologically relevant or oxidative DNA damage by the hydroxyl radical is the primary cause of cell death under oxidative stress conditions (Macomber et al., 2007). Supercoiled plasmid DNA was prone to oxidation by Fenton reactants as a result of the formation of open circular and linear forms which indicate the double-strand breaks. Figure 1 shows that BWE had greater ability to protect DNA from open circular to linear (Figure 1, lane 5) as compared to the other samples tested. In contrast with the blank control (Figure 1, lane 3), trolox had no obvious effect on the inhibition activity on hydroxyl radical induced plasmid pBR 322 DNA damage (Figure 1, lane 6), but when trolox were mingled with AIEs, their inhibition activities increased and trolox with BWE presented a higher ability of restraining DNA breakage than trolox with AWE (Figure 1; lanes 8 and 7, respectively).

### Inhibitory effects on supercoiled DNA breakage induced by peroxynitrite

It has been demonstrated that ONOO<sup>-</sup> may trigger DNA strand breaks in all kinds of systems (Jia et al., 2009; Cao and Li, 2004). As shown in Figure 2, incubation of the plasmid DNA with ONOO<sup>-</sup> at 37°C for 60 min resulted in an increased formation of open circular form of DNA. The AIEs were good inhibitors in preventing the formation of open circular DNA while Trolox displayed the same tendency as previous study that the inhibition activities increased when trolox was mixed with AIEs; no significant differences were observed between trolox with BWE and trolox with AWE.



**Figure 2.** Inhibitory effects of AIEs and trolox in preventing DNA strand cleavage by  $\text{ONOO}^-$ . Lane 1, HindIII  $\lambda$ -digest DNA size markers; lane 2, intact pBR322 DNA (no  $\text{ONOO}^-$ ); lane 3,  $\text{ONOO}^-$  induced DNA scission (no extracts); lane 4,  $\text{ONOO}^-$  + AWE; lane 5,  $\text{ONOO}^-$  + BWE; lane 6,  $\text{ONOO}^-$  + trolox; lane 7,  $\text{ONOO}^-$  + AWE + trolox; lane 8,  $\text{ONOO}^-$  + BWE + trolox.

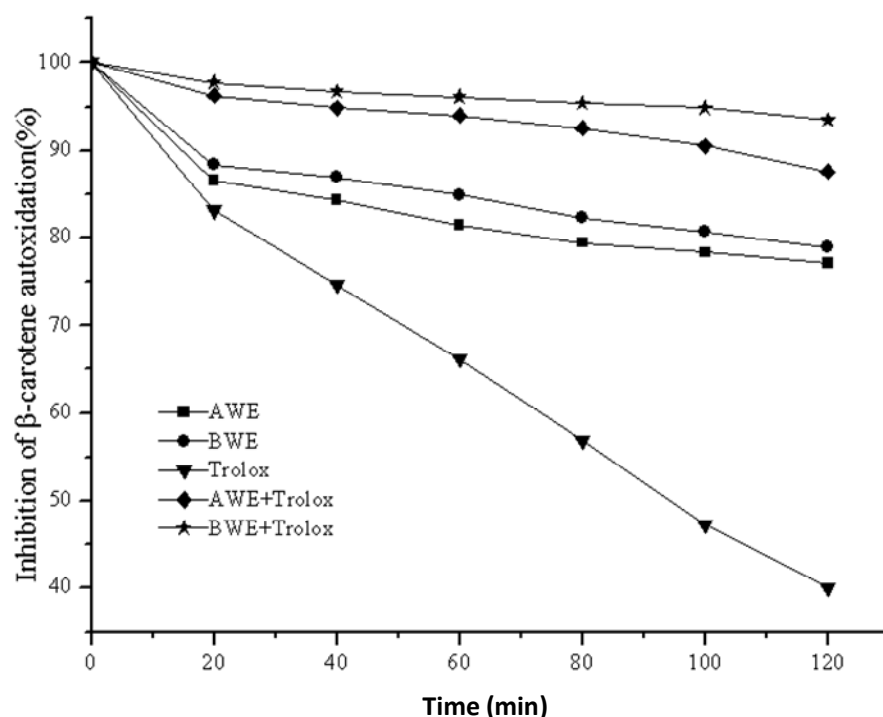


**Figure 3.** Effects of AIEs and trolox on decolorization of ABTS radical cation. The percentage inhibition was plotted against the concentration of sample. All data are expressed as mean  $\pm$  S.D. ( $n = 3$ ).

### The ABTS radical cation decolorization assay

Total antioxidant activity of AIEs and trolox were calculated from the decolorization of  $\text{ABTS}^+$ , which was measured spectrophotometrically at 734 nm to evaluate free radical scavenging properties. The results of the investigation are shown in Figure 3. For all the phenolic compounds tested, inhibitory activity increased with

increasing concentrations. At the same concentration tested, BWE showed the highest inhibitory activity towards  $\text{ABTS}^+$ . The interaction with the extract and trolox suppressed the absorbance of the  $\text{ABTS}^+$  radical cation. The inhibition activities of BWE with trolox and AWE with trolox were higher than that of trolox alone, but lower than AIEs which might indicate that the AIEs increased the antioxidant activity of trolox.



**Figure 4.** The inhibition ratio of linoleic acid oxidation by extracts measured  $\beta$ -carotene-linoleic acid assays.

### Assay for $\beta$ -carotene autoxidation

The effect of AIEs trolox on the coupled oxidation of  $\beta$ -carotene and linoleic acid was examined.  $\beta$ -Carotene, a polyene pigment, was oxidized easily and had a faded color which could be measured at 470 nm. In this assay, antioxidant capacity is determined by measuring the inhibition of the conjugated diene hydroperoxides arising from linoleic acid oxidation (Alessandra braca et al., 2003). It is generally deemed that the inhibition of lipid peroxidation by antioxidants may be due to their free radical scavenging activities which prevent the decolorization of  $\beta$ -carotene. As shown in Figure 4, the inhibition of  $\beta$ -carotene autoxidation by AIEs and trolox all decreased with the increase of reaction time and trolox nearly declined linearly. The results demonstrate that the antioxidant activities of the test samples were BWE + trolox > AWE + Trolox > BWE > AWE > trolox, respectively, which indicate that the AIEs could increase the antioxidant activity of trolox, and the effect of BWE was more significant than AWE.

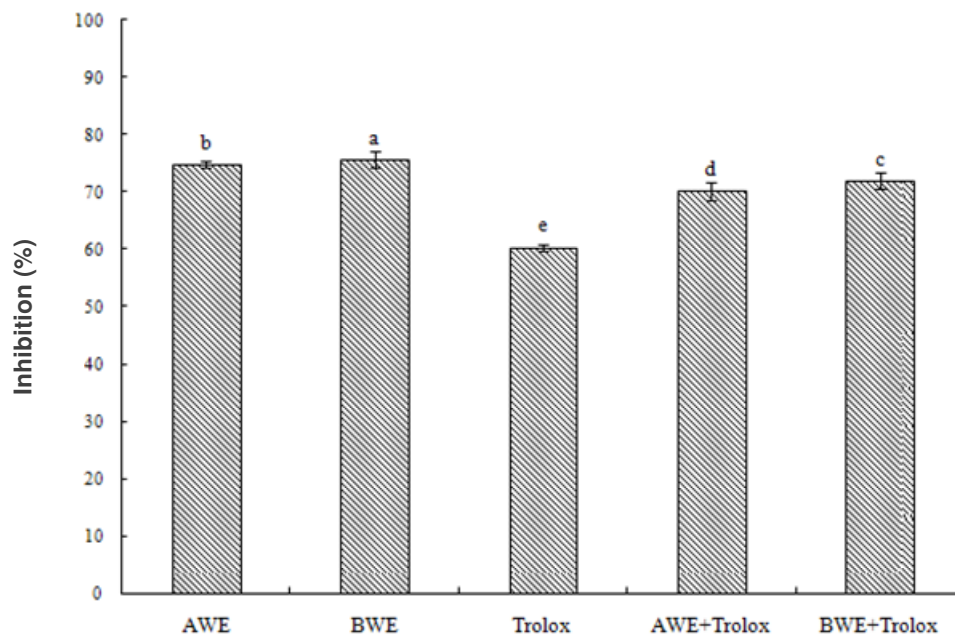
### Determination of 5-lipoxygenase inhibiting activity of AIEs

The percentage inhibition of 5-lipoxygenase activity by two AIEs, trolox and their interaction with each other are shown in Figure 5. At the same concentration tested, BWE showed the highest inhibiting activity towards 5-

lipoxygenase and trolox showed the lowest activity in the three samples. When AIEs and trolox were added together, keeping the same volume with single sample, the inhibiting activity of trolox increased, indicating that AIEs and trolox have a distinctly synergistic antioxidant efficiency that increase the antioxidant activity of trolox. Moreover, the synergistic efficiency of BWE and trolox was higher than AWE and trolox. It has been reported that the inhibitory activity of phenolic compounds towards 5-lipoxygenase depends on the number of hydroxyl substituents, and the phenolic compounds might bind with 5-lipoxygenase via hydrophobic interactions or hydrogen bonds, leading to the conformational changes of 5-lipoxygenase (Sadik et al., 2003; Schurink, 2007; Maqsood and Benjakul, 2010). In this study, the AIEs displayed high 5-lipoxygenase inhibiting activity which may be due to their abundant hydroxyl substituents of phenolic compounds which could increase the antioxidant activity of trolox.

### Conclusion

Reactive oxygen and nitrogen species were implicated in DNA oxidative damage and then caused aging and cancer. Our results of ABTS radical cation decolorization assay, and autoxidation of  $\beta$ -carotene assay may demonstrate that the AIEs were good free radical scavengers due to their phenolic compounds. The AIEs displayed powerful protective effect on pBR322 DNA damage



**Figure 5.** Lipoxigenase inhibitory activity of AIEs and trolox. Bars represent the standard deviation (n = 3). Different small letters denote significant differences (P < 0.05).

caused by hydroxyl radical and peroxynitrite radical because of its free radical scavenging activity. The AIEs also have strong 5-lipoxygenase inhibiting activity and synergistic antioxidant activity with trolox. The results provide evidence for the phenolic dependent biological activity of areca inflorescence and may be widely used in the industry of pharmaceuticals, high-grade cosmetics, health products and food.

## ACKNOWLEDGEMENTS

This work was supported by Hainan Natural Science Found (project no. 310101) and Hainan Key Scientific and Technological Projects (project no. 090138).

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